Plant Mol Biol (2007) 64:387–395
DOI 10.1007/s11103-007-9160-0

Activation of the imprinted Polycomb Group *Fie1* gene in maize endosperm requires demethylation of the maternal allele

Pedro Hermon · Kanok-orn Srilunchang · Jijun Zou · Thomas Dresselhaus · Olga N. Danilevskaya

Received: 30 October 2006 / Accepted: 1 March 2007 / Published online: 17 April 2007
© Springer Science+Business Media B.V. 2007

Abstract Imprinting refers to the epigenetic regulation of gene expression that is dependent upon gene inheritance from the maternal or paternal parent. Previously, we have identified two maize homologs of the single *Arabidopsis* Polycomb Group gene *FIE*. Here, we report on the expression pattern of these genes in individual gametes before and after fertilization, and on the role of DNA methylation in determining the maternal expression of the *Fie1* gene. We found that *Fie1* is neither expressed in the sperm, egg cell nor central cell before fertilization. Activation of the *Fie1* maternal allele occurs around two days after pollination (DAP) in the primary endosperm and peaks at 10–11 DAP coinciding with endosperm transition from mitotic division to endoreduplication. In contrast, *Fie2* is expressed in the egg cell and more intensively in the central cell similar to Arabidopsis *FIE*, which strongly supports the hypothesis that it functions as a repressor of endosperm development before fertilization. Using MSRE-PCR and bisulfite sequencing, we could show that the methylated inactive state is the default status of *Fie1* in most tissues. In the endosperm the paternal *Fie1* allele remains methylated and silent, but the maternal allele appears hypomethylated and active, explaining mono-allelic expression of *Fie1* in the endosperm. Taking together, these data demonstrate that the regulation of *Fie1* imprinting in maize is different from Arabidopsis and that *Fie1* is likely to have acquired important novel functions for endosperm development.

Keywords Imprinting · DNA methylation · Fertilization · Endosperm · Gametes · PcG

Electronic supplementary material The online version of this article (doi:10.1007/s11103-007-9160-0) contains supplementary material, which is available to authorized users.

P. Hermon · J. Zou · O. N. Danilevskaya
Pioneer Hi-Bred International Inc, 7250 NW 62nd Ave, Johnston, IA, USA

K.-o. Srilunchang · T. Dresselhaus (✉)
Cell Biology & Plant Physiology, University of Regensburg, Universitätsstrasse 31, 93053 Regensburg, Germany
e-mail: thomas.dresselhaus@biologie.uni-regensburg.de

Introduction

A subset of animal and plant genes is expressed in a parent-of-origin-specific manner either from the paternal or maternal allele. This phenomenon, called imprinting, was first discovered in maize as a maternal control of the kernel aleurone color (Kermicle 1970). Later, imprinting was described in mice through pronuclear transplantation experiments (Surani and Barton 1983; Reik and Walter 2001). To date, more than 80 imprinted genes have been described in mammals (Morison et al. 2005).

In plants imprinting has been found so far only in the endosperm, a terminal nutritive tissue that develops after double fertilization from the fertilized central cell. The endosperm provides nutrients for developing embryos in dicots and for germinating seedlings in monocots (Kranz et al. 1998; Walbot and Evans 2003). A dozen or so imprinted genes have been discovered in plants, of which the majority are maternally expressed and paternally silenced in the endosperm (Alleman and Doctor 2000; Baroux et al. 2002; Gehring et al. 2004a, b). So far, only the *Arabidopsis* MADS-box gene *PHERES1* was shown to be expressed paternally due to the repressing activity of the Polycomb Group (PcG) protein, MEDEA (Köhler et al. 2003a, b, 2005).

Plant PcG proteins are important components of parent-of-origin control of gene expression and have been

reported recently to be involved also in the self-regulation of imprinting (Baroux et al. 2006; Gehring et al. 2006; Jullien and Katz et al. 2006; Jullien and Kinoshita et al. 2006). PcG proteins form complexes that are able to silence genes and maintain their silencing over many cell divisions by a mechanism that relies mainly on histone modifications at the repressed locus (Delaval and Feil 2004). The *Drosophila* Enhancer of Zeste-Extra Sex Combs [E(Z)-ESC] PcG complex, for example, functions as a repressor of a number of homeotic genes (Sathe and Harte 1995). The E(Z)-ESC PcG complex appears to be conserved, but more complex in plants (Grossniklaus and Schneitz 1998; Grossniklaus et al. 1998; Luo et al. 1999; Ohad et al. 1999; Springer et al. 2002; Jullien and Katz et al. 2006; Jullien and Kinoshita et al. 2006). In *Arabidopsis* the Fertilization-independent seed (FIS) complex that controls seed development is composed of Medea (MEA), Fertilization-independent endosperm (FIE), Fertilization-independent seed2 (FIS2) and Multicopysupressor of IRA 1 (MSI1), which are homologs of the Drosophila E(Z), ESC, Suppressor of Zeste12 and CAF1/P55 PcG proteins, respectively (Grossniklaus and Schneitz 1998; Grossniklaus et al. 1998; Luo et al. 1999; Ohad et al. 1999; Köhler et al. 2003a, b). *MEA* and *FIS2* are imprinted genes and are expressed maternally in the endosperm (Grossniklaus and Schneitz 1998; Kinoshita et al. 1999; Jullien and Katz et al. 2006; Jullien and Kinoshita et al. 2006). These two genes as well as the imprinted gene *FWA*, which displays an endosperm-specific expression, have been studied intensively to elucidate the mechanism of imprinting in *Arabidopsis* (Kinoshita et al. 2004; Baroux et al. 2006; Gehring et al. 2006; Jullien and Katz et al. 2006; Jullien and Kinoshita et al. 2006). It was shown that the maintenance of *FWA* and *FIS2* imprinting depends on DNA methylation (Kinoshita et al. 2004; Jullien and Katz et al. 2006; Jullien and Kinoshita et al. 2006). In contrast, the *MEA* paternal allele is silenced by the MEA-FIE PcG complex due to histone methylation and thus is independent of DNA methylation (Baroux et al. 2006; Gehring et al. 2006; Jullien and Katz et al. 2006; Jullien and Kinoshita et al. 2006). However, *MEA*, *FWA* and *FIS2* imprinting share a common feature such as the activation of maternal alleles in the central cell due to demethylation by Demeter (DME) DNA glycosylase before fertilization (Choi et al. 2002; Kinoshita et al. 2004; Gehring et al. 2006; Jullien and Katz et al. 2006; Jullien and Kinoshita et al. 2006). After fertilization, the maternal allele remains transcriptionally active in the endosperm, whereas the paternal allele remains silent as it was in the sperm. Because the endosperm is a terminal tissue and is not transmitted to the next generation, the epigenetic state of the imprinted genes does not require re-setting of the imprinting marks and a model for the one way control of imprinting in *Arabidopsis* was proposed (Kinoshita et al. 2004).

Despite the original discovery of imprinting in maize, the paucity of information about the molecular mechanisms of imprinting is available in this species. Maternal demethylation of certain alleles of zein and α-tubulin genes have been reported in the maize endosperm (Lund and Ciceri et al. 1995; Lund and Messing et al. 1995; Alleman and Doctor 2000). Extensive maternal hypomethylation was detected in the maize endosperm by a PCR-based genomic scan (Lauria et al. 2004). However, DNA methylation of locus-specific imprinted genes has not been reported in maize until recently (Gutierrez-Marcos et al. 2006). Previously, we identified two *FIE* homologs in maize, *Fie1* and *Fie2*, that show distinct expression patterns and imprinting during kernel development (Springer et al. 2002; Danilevskaya et al. 2003). *Fie1* expression is restricted to the endosperm and shows mono-allelic expression from the maternal allele, whereas, *Fie2* is broadly expressed in many tissues and shows bi-allelic expression in the embryo and the endosperm at later stages (Danilevskaya et al. 2003; Gutierrez-Marcos et al. 2003). Based on the pattern of expression we proposed that *Fie1* and *Fie2* may have evolved divergent functions in maize. In this study, we examined *Fie1* and *Fie2* expression in the isolated gametes before fertilization, and in zygotes and developing kernels at the early stages after fertilization. We also investigated DNA methylation of *Fie1* and *Fie2* and report the demethylation of the maternal *Fie1* allele, but not of the paternal allele after fertilization and the silencing by DNA metylation of *Fie1* in other tissues including the embryo and endosperm.

Materials and methods

Plant material

Reciprocal crosses were performed between maize inbred lines B73 and Mo17. Embryo and endosperm tissues were dissected from kernels at 14 days after pollination (DAP). Pericarp was removed from the kernels and was not included in DNA extraction. Tissues were frozen in liquid nitrogen and stored at –80°C. Pollen and leaf tissue were obtained from mature B73 or Mo17 plants. Inbred line A188 was used for isolation of individual gametes.

DNA isolation and MSRE-PCR

Genomic DNA was isolated from 10 mg to 20 mg of frozen embryo and endosperm tissues using Puregene^{TM} DNA isolation components (Gentra Systems, Minneapolis, MN). About 0.5 μg of genomic DNA was digested at 37°C

overnight in a 50 μl reaction with 25 U of either *Hpa*II or *Msp*I (New England Biolabs, Beverly, MA) for MSRE-PCR (Liang et al. 2004). After an overnight incubation, 25 additional units of the appropriate restriction enzyme were added and the reaction was incubated for 2 h at 37°C. PCR amplification was performed using Expand Long Template DNA polymerase (Roche, Germany). About 2 μl of the restriction enzyme reaction was used for PCR amplification in a 50 μl volume. The PCR conditions were 95°C for 5 min followed by 35 cycles at 95°C for 45 s, 60°C for 45 s, 72°C for 1 min and a final extension of 72°C for 10 min.

Bisulfite sequencing

Bisulfite treatment (Engemann et al. 2001) was performed with the EZ DNA Methylation Kit™ (Zymo Research, Orange, CA). In a 50 µl reaction, 1 µg of genomic DNA was treated according to the manufacturer's recommendations. After bisulfite treatment, PCR was performed using gene specific primers for the bisulfite converted DNA sequence (Details in Suppl. Materials). The PCR conditions used were the same as outlined for MSRE-PCR. PCR products were subcloned into pCR®4-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced with M13F and M13R primers.

Single Cell RT-PCR (SC RT-PCR)

Cells of the female gametophyte before and after fertilization as well as sperm cells were isolated from the maize inbred line A188 as described by Cordts et al. (2001). SC RT-PCR was performed according to Richert et al. (1996) with one modification. A multiplex reverse transcription reaction was conducted on each cell either with reverse R-fie1 and R-fie2 primers for *Fie1* and *Fie2*, respectively, in addition to the reverse *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) primer Gap2. Generated cDNA was split into two equal amounts to amplify *Fie* and *GAPDH* transcripts separately during 40 PCR cycles using primers described in Table S1. Gels were blotted and hybridized as described (Dresselhaus et al. 1999) with radio-labeled *Fie1* and *Fie2*-specific probes.

Real-time quantitative RT-PCR (qRT-PCR)

Developing kernels were collected every 24 h from the ear base harvested from the B73 inbred pollinated by the Mo17 inbred in the field. Total RNA was isolated from whole kernels using TRIzol® Reagent with Phase Lock Gel-Heavy (Eppendorf North America, Inc., Molecular Research Center, Inc.). Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA) and reverse-transcribed by using the Taqman Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The final RNA concentration in the cDNA synthesis reaction was 30 ng/μl. The absence of genomic DNA contamination was confirmed by the PCR without the reverse transcriptase. To ensure gene-specific amplification, primers and 5' FAM labeled LNA probes (Suppl. Table S2) were designed against 3' UTR of *Fie1* and *Fie2* using Universal ProbeLibrary (Roche Diagnostics, Indianapolis, IN). Primers and the 5' VIC labeled MGB probe (Table S2) for *β-Actin* were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). The expression of each gene was assayed in triplicate in a total volume of 20 μl containing 1× Taqman master mix, 300 nM forward and reverses primers, 100 nM probes and 3 μl of the 1:4 diluted cDNA. The PCR thermal cycling parameters were 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All assays were run on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Transcript levels of *Fie1* and *Fie2* were measured relatively to the endogenous reference *β-Actin* with the Ct method as described by the manufacturer. The PCR amplification efficiency was determined by measuring a series of input cDNA concentrations. The PCR efficiency for *Fie1, Fie2* and *β-Actin* was 1.01, 1.03 and 1.02, respectively.

Results

The maternal *Fie1* allele is activated in the endosperm after fertilization whereas *Fie2* is expressed in both gametes and zygotes

As shown previously, *Fie1* transcript was detected exclusively in the endosperm where it is expressed from the maternal allele. *Fie2* is expressed in a broad set of tissues including ovules before fertilization (Danilevskaya et al. 2003; Gutierrez-Marcos et al. 2003). However, *Fie1* and *Fie2* expression was never analyzed in gametes. To address this issue, transcription of *Fie1* and *Fie2* was examined in manually isolated gametes and zygotes by single cell RT-PCR (SC RT-PCR) (Richert et al. 1996; Cordts et al. 2001). *Fie1* mRNA was neither detected in the individual gametes nor in zygotes 12 h after in vitro pollination, which is around 6 h after fertilization (Fig. 1A). In contrast, *Fie2* transcript is present in all gametic cells with the highest transcript level in the central cell (Fig. 1B). To detect the onset of *Fie1* activation in the endosperm, developing kernels were collected every 24 h after pollination and analyzed by quantitative real-time RT-PCR (qRT-PCR) for *Fie1* and *Fie2* expression. *Fie1* transcript was at background level in the ovules and in developing

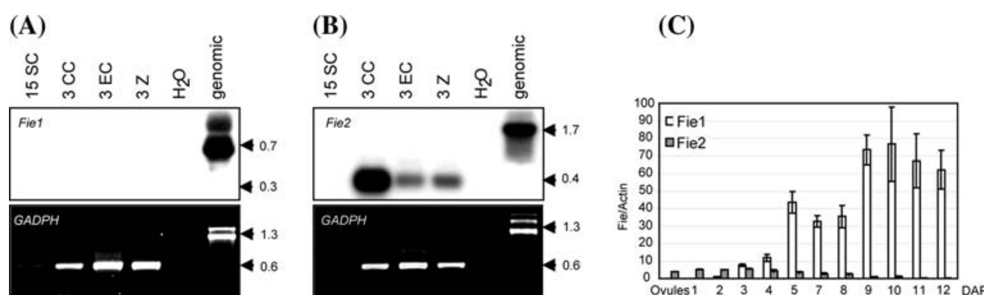

Fig. 1 *Fie1* and *Fie2* expression in gametes and developing kernels. (**A**) RT-PCR analysis of *Fie1* and (**B**) *Fie2* expression in 15 sperm cells (SC), 3 central cells (CC), 3 egg cells (EC), and 3 zygotes (Z) collected 12 h after in vitro pollination (around 6 h after fertilization). *Fie* PCR products were blotted and hybridized with a gene-specific probe. DNA fragment sizes (kb) are indicated by arrows. This experiment was repeated twice to confirm the obtained result. (**C**) Quantification of the *Fie1* and *Fie2* mRNAs in developing kernels by qRT-PCR. Relative amounts were calculated and normalized with respect to *actin* transcript levels (=100%). Data shown represent mean values obtained from three independent amplification reactions, and the error bars indicate the standard error of the mean. The original numbers are shown in Suppl. Table S1

kernels at 1 DAP. *Fie1* mRNA was first detected around 2 DAP (Fig. 1C and Suppl. Tables S1 and S2). Because fertilization occurs around 16–24 h after in vivo pollination (Kiesselbach 1999), we conclude that *Fie1* activation takes place approximately 24–32 h after fertilization. The highest *Fie1* mRNA level was detected in kernels at 10–11 DAP and gradually decreased at later stages (Fig. 1C). In contrast, *Fie2* transcript was present in mature ovules and its level slightly increased at 1–4 DAP, but decreased at later stages. Overall, *Fie2* mRNA level was significantly lower than *Fie1*. At 10 DAP, expression of *Fie1* is about 80 times higher than that of *Fie2*.

Fie1 is methylated in most tissues whereas *Fie2* is not methylated

To examine the role of DNA methylation in determining the expression pattern of the *Fie1* (GenBank Accession #AY150645) and *Fie2* (GenBank Accession #AY150646) genes, we analyzed their methylation status in different tissues. Methylation sensitive restriction enzyme digestion PCR (MSRE-PCR) analysis with the isoschizomers *Hpa*II and *Msp*I was performed to assess the methylation pattern across the two genes (Liang et al. 2004). Both enzymes recognize CCGG sites but *Hpa*II does not cut DNA if either cytosine is methylated. *Msp*I does not cut DNA if the external cytosine is methylated. *Fie1* contains seven CCGG sites distributed throughout the gene and *Fie2* contains ten CCGG sites clustered in upstream and at exon 1 (Fig. 2A and Suppl. Table S3). Using both enzymes for MSRE-PCR analysis, we found that CCGG sites at exon 1 and exon 7 were methylated in the embryo, endosperm and leaf DNA (Fig. 2B). CCGG sites at exons 11–13 showed a low level of methylation. DNA methylation was not detected in *Fie2* (Fig. 2B).

Bisulfite sequencing (Engemann et al. 2001) was applied to quantify *Fie1* and *Fie2* DNA methylation at CpG and CpNpG sites in the embryo, endosperm, pollen and leaf tissues. The promoter region was of particular interest because it lacks *Hpa*II/*Msp*I sites. The promoter, exon 1 and the region including exons 6–8 of *Fie1* are shown in Fig. 3A. Endosperm DNA revealed a low level of methylation ranging from 20% to 40% at CG sites and from 14% to 16% at CNG sites (Fig. 3A and Suppl. Table S4) in all segments analyzed including the promoter. In contrast, *Fie1* DNA from embryo, pollen and leaves was methylated up to 58–97% at CG-sites and 50–75% at CNG sites (Fig. 3A and Suppl. Table S4). Combining MSRE-PCR and bisulfite sequencing allowed the conclusion that the *Fie1* gene is methylated across the promoter region and approximately half of the coding region up to exon 8. Significant cytosine methylation was not detected at the *Fie2* gene (Fig. 3B).

Maternal *Fie1* allele is hypomethylated in the endosperm

To discriminate maternal and paternal alleles in bisulfite treated DNA molecules, we took advantage of two SNPs in exon 1 in inbred lines B73 and Mo17 (Fig. 4A). Due to the preferential amplification of the maternal molecules from the endosperm DNA (Fig. 3A) we have sequenced 116 clones amplified from exon 1 using endosperm DNA from reciprocal B73 × Mo17 crosses. 102 clones were of maternal and 14 of paternal origin. We found that maternal endosperm molecules were hypomethylated (2.8% methylated cytosines at CG sites and 3.3% at CNG) compared to corresponding paternal endosperm molecules (65.7% at CG and 49.2% at CNG). Maternal DNA isolated from embryos displayed a high methylation content (64.0% at CG and 56.7% at CNG) similar to corresponding paternal DNA regions (70.0% at CG, 63.9% at CNG) (Fig. 4B and Suppl. Table S4). Because the maternal endosperm alleles have been preferentially amplified, we designed an additional experiment to confirm methylation at the

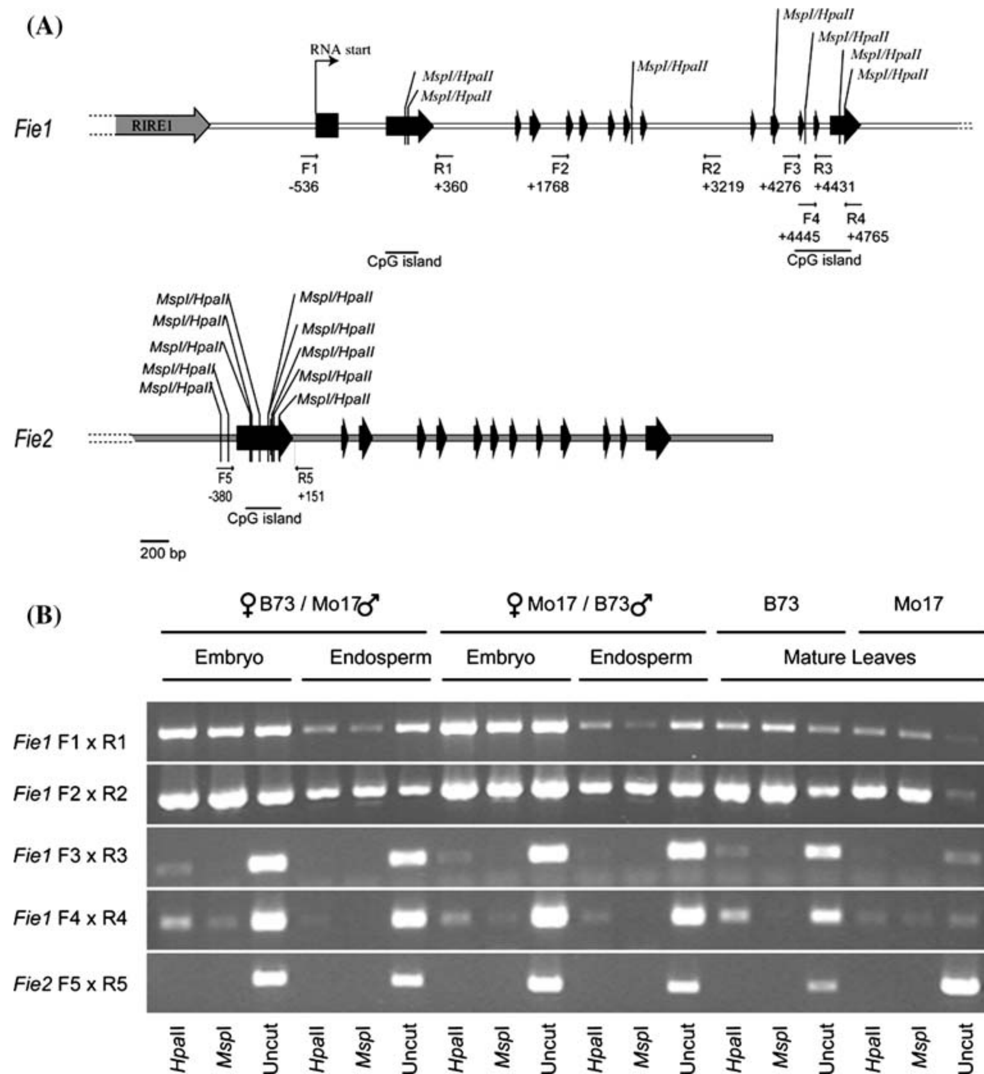

Fig. 2 Methyl-sensitive restriction-enzyme-dependent (MSRE) PCR of *Fie1* and *Fie2*. (**A**) Genomic maps of *Fie1* (GenBank accession #AY150645) and *Fie2* (GenBank accession #AY150646) are shown with *Msp*I/*Hpa*II restriction sites. Exons are depicted by black arrows. Introns are shown with lines. Retrotransposon *RIRE* is depicted by the shaded arrow. Gene specific primers, shown with small arrows, were used to amplify four regions of *Fie1* and one region of *Fie2* containing *Msp*I/*Hpa*II sites. Primers are marked with location relative to the ATG start codon. (**B**) Agarose gel with PCR products of the five analyzed regions of the *Fie* genes. Genomic DNA was isolated from embryo and endosperm tissue from reciprocal crosses of inbred lines B73 and Mo17 (female shown first). DNA was treated with *Msp*I or *Hpa*II restriction enzyme prior to amplification. The presence of PCR products indicates methylation of *Msp*I/*Hpa*II sites within the region. Undigested DNA was used as a positive control

paternal allele. Embryo and endosperm DNA from reciprocal crosses of B73 and Mo17 were digested to completion with *Hpa*II and amplified across *Hpa*II sites and SNPs in exon 1 with gene-specific primers extended with T3 and T7 adapters (Fig. 4A). PCR fragments obtained were directly sequenced with T3 and T7. Sequencing chromatograms of PCR products from digested and undigested embryo DNA showed the presence of SNPs from both parents, B73 and Mo17 (Fig. 4C), indicating that both parental alleles are methylated in the embryo. Conversely, the chromatograms of PCR products generated from digested endosperm DNA showed the presence of paternal SNPs but a complete absence of maternal SNPs (Fig. 4D). Undigested endosperm DNA showed a mixture of traces from both parents. These results confirm that the paternal *Fie1* allele is methylated in the endosperm and therefore protected from digestion by *Hpa*II. The maternal alleles are unmethylated and can be digested by *Hpa*II. In summary, the maternal *Fie1* allele is hypomethylated in the endosperm but methylated in the embryo. The expression of *Fie1* is thus strongly correlated with a loss of DNA methylation of the maternal allele in the endosperm. The paternal allele is methylated in the endosperm and all other tissues investigated.

Discussion

The maize genome contains two homologous *Fie* genes which have distinct expression pattern (Danilevskaya et al. 2003). *Fie1* is a highly regulated gene, expressed exclusively in the endosperm, where its expression is controlled by imprinting, resulting in mono-allelic expression of the maternal but not the paternal allele. In contrast, *Fie2* shows bi-allelic expression in many tissues including embryo and endosperm at later stages of development. The Arabidopsis *FIE* gene functions as a repressor of endosperm development in the central cell

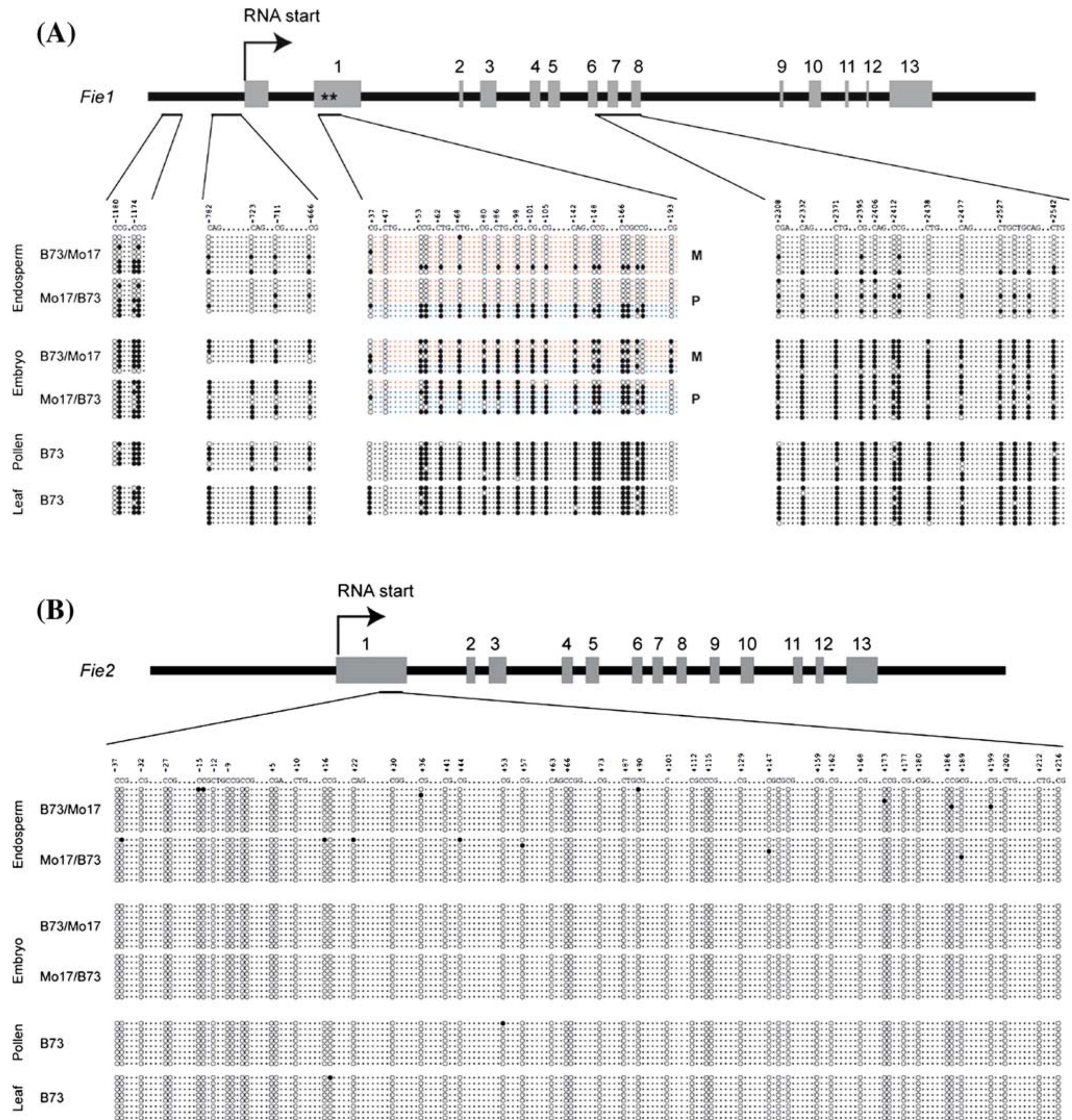

Fig. 3 Bisulfite DNA methylation pattern of *Fie1* and *Fie2* in different maize tissues. (**A**) Genomic structure of *Fie1* is shown above methylation profiles. Exons are depicted with shaded boxes. Introns are shown by solid black lines. The transcription start point is marked with an arrowhead and two SNPs in exon 1 between parental inbred lines B73 and Mo17 are marked with asterisks. Regions used for bisulfite sequencing are underlined. Percent methylation at CG, CNG and asymmetric sites in endosperm, embryo, pollen and leaf is indicated. (**B**) Genomic structure and methylation profile of *Fie2*. Description as in (A). Detailed information about numbers and clones analyzed and primers used are provided in Suppl. Tables S3 and S4

prior to fertilization (Ohad et al. 1999) and seems to be involved in the regulation of the ontogenic sequence of endosperm development after fertilization (Ingouff et al. 2005). To elucidate functions of maize *Fie* genes prior to fertilization, we examined their expression pattern in manually isolated gametes from maize. Neither gene is expressed in the sperm. Out of both *Fie* genes, only *Fie2* transcript was detected in the central cell supporting its putative function as a repressor of endosperm development before fertilization. At a lower level, *Fie2* tran-

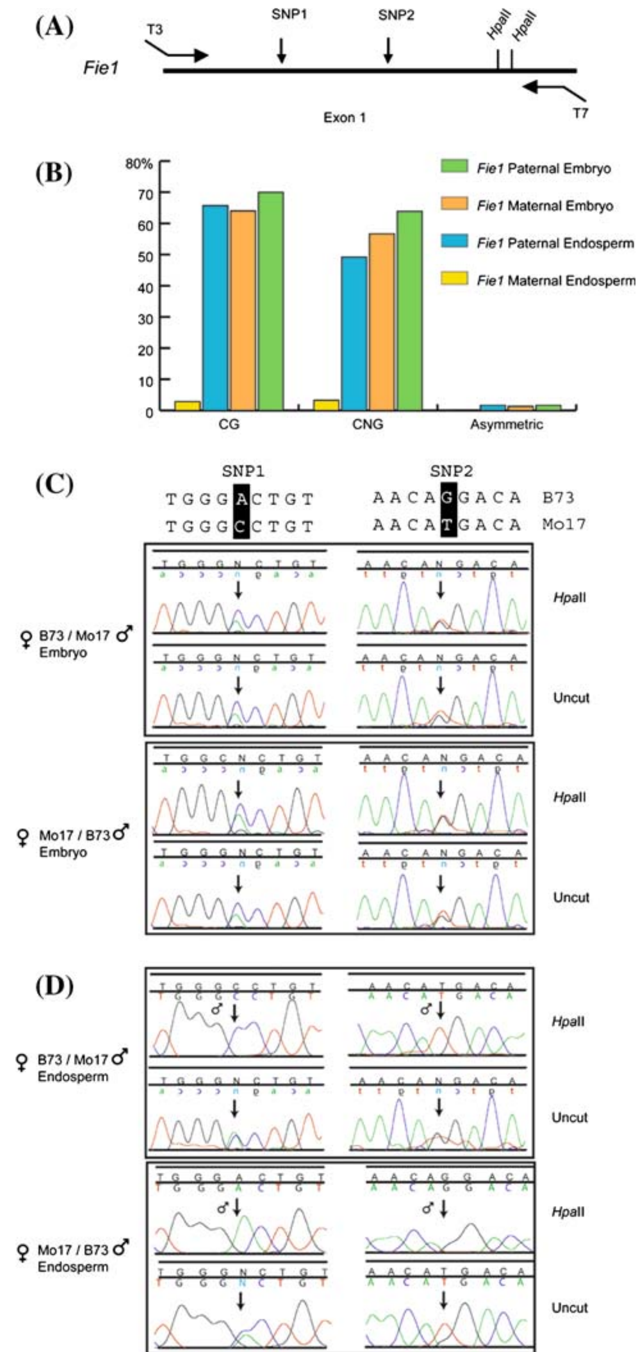

Fig. 4 Demethylation of the maternal *Fie1* allele in the endosperm. (**A**) Parental *Fie1* alleles in reciprocal crosses were distinguished by two SNPs in exon 1 between inbred lines B73 and Mo17, respectively. Gene specific primers with T3 or T7 extensions were designed around SNPs and two linked *Hpa*II sites for direct sequencing. (**B**) Percent methylation at CG, CNG and asymmetric sites of the *Fie1* maternal and paternal alleles from the endosperm and embryo (Suppl. Table S4). Alleles were discriminated by SNPs in the exon 1. (**C, D**) Chromatograms of PCR products amplified from *Hpa*II-digested and undigested embryo (C) and endosperm (D) DNA as indicated. Arrows point to the SNPs on chromatogram traces

scription was also detected in the egg cell and after fertilization in the zygote and developing endosperm. This expression pattern might be attributed to additional functions of *Fie2* during kernel development similar to that of Arabidopsis *FIE*. Knock-out mutants should now be analyzed to study *Fie2* functions. However, such mutants are not available yet.

The absence of *Fie1* transcript in the central cell and the egg cell indicates that this gene does not play a role in the regulation of pre-fertilization events in the embryo sac. Rather it might have very specific function(s) during endosperm development. Endosperm development in maize and other cereals is characterized by distinct changes of the cell cycle pattern. The primary endosperm nucleus, resulting from fusion of a sperm with the two polar nuclei of the central cell, divides mitotically within 3–5 h after fertilization without cytokinesis forming a multinucleate syncytium (Kiesselbach 1999). Cell wall deposition is activated around 3 DAP and is completed at 4–5 DAP. Mitotic proliferation continues up to 10–15 DAP until the cell cycle switches to endoreduplication (Kowles and Phillips 1988). The transition from mitotic divisions to endoreduplication is thought to be controlled by parental imprinting (Dilkes et al. 2002; Leblanc et al. 2002). Expression of *Fie1* begins approximately 24–32 h after fertilization reaching the highest level at 10–11 DAP coinciding with the described switch of the cell cycle from mitotic divisions to endoreduplication. As a PcG protein, *Fie1* might regulate imprinting of other genes and it is therefore tempting to speculate that *Fie1* might have a function in the maternal control of the transition to endoreduplication in the maize endosperm. Rice and sorghum genomes also contain two *FIE* homologues (Lai et al. 2004), suggesting that *Fie* genes in cereals might have evolved distinct functions compared to the single Arabidopsis *FIE* gene. It is interesting now to study whether rice and sorghum *Fie1*-like genes are also regulated by imprinting as is the case for the maize *Fie1* gene. This would indicate conserved functions in the grasses.

The distinct feature of the *Fie1* gene is its mono-allelic expression from the maternal allele in the endosperm at all stages of development (Danilevskaya et al. 2003). *Fie2* shows bi-allelic expression in the embryo and in the endosperm at later stages. However, in the early endosperm at 6 DAP, *Fie2* has been shown to be expressed maternally (Danilevskaya et al. 2003; Gutierrez-Marcos et al. 2003). To understand the role of DNA methylation in tissue-specific expression and imprinting, we have examined the methylation status of both *Fie* genes. *Fie1* was found to be methylated in all tissues tested, which is consistent with its restricted expression pattern and indicates that the

methylated silent state is the default for *Fie1*. DNA methylation was not detected in the *Fie2* gene, which is consistent with its broad expression in many tissues. However, recently *Fie2* methylation of the paternal allele was observed in 6 DAP endosperm indicating that the transient methylation of this gene takes place only during early stages of endosperm development (Gutierrez-Marcos et al. 2006), as the gene is no longer methylated at 14 DAP (this report).

Using bisulfite sequencing, we found a high level of *Fie1* methylation at the promoter region, exon 1 and exons 6–8 in DNA samples isolated from embryos, pollen and leaves. A lower level of DNA methylation was detected in the endosperm, where only the maternal *Fie1* alleles are expressed. Our results further showed that the maternal *Fie1* allele is hypomethylated in the endosperm, but the paternal allele is hypermethylated. In DNA extracted from embryos, both maternal and paternal alleles are methylated at the same level.

This methylation pattern strongly correlates with *Fie1* expression. *Fie1* is methylated and silent in most tissues except the endosperm where the maternal allele is demethylated and transcribed.

Because *Fie1* transcript was not detected in the central cell, it was unclear when demethylation of the *Fie1* gene actually occurs. According to a recent study by Gutierrez-Marcos et al. (2006), *Fie1* is methylated in the sperm and the egg cell, but hypomethylated in the central cell. Thus the demethylation of the *Fie1* gene occurs before fertilization in the central cell. Despite its demethylation in the central cell, *Fie1* becomes transcriptional active only in the primary endosperm after fertilization suggesting that demethylation is necessary but not sufficient for its activity. Endosperm-specific factors are apparently required to activate transcription of the maternal *Fie1* gene in the endosperm. Demethylation and transcriptional activation of the maternal alleles of *MEA* (Xiao et al. 2003; Kinoshita et al. 2004), *FWA* (Kinoshita et al. 2004) and *FIS2* (Jullien and Katz et al. 2006; Jullien and Kinoshita et al. 2006) occur during female gametogenesis in the central cell by the antagonistic activity of MET1 methyltransferase and DME, a DNA glycosylase with a 5-methylcytosine excising activity (Choi et al. 2002; Gehring et al. 2006). *DME* is specifically expressed in the central cell preceding fertilization, erasing methylation marks set up by *MET1* on *MEA*, *FWA* and *FIS2* (Xiao et al. 2003; Kinoshita et al. 2004; Jullien and Katz et al. 2006; Jullien and Kinoshita et al. 2006). The DME-like genes might play similar roles by erasing methylation marks on imprinted genes in maize as well.

Until recently, the molecular mechanisms of imprinting in plants have most extensively been studied in *Arabidopsis*. These studies revealed two types of imprinted genes. *MEA*, for example, shows bi-allelic expression in many tissues except the endosperm, where the gene is maternally expressed. Other genes, like *FWA* and *FIS*, are silent and methylated in all tissues except the endosperm, suggesting that methylation is the default state. The *Fie1* default state is also methylated and the maternal allele is activated due to its demethylation in the central cell prior to fertilization. We found that *Fie1* is methylated across the extended ~4 kb segment including the promoter and coding region up to exon 7. This is significantly deviating from imprinted genes in *Arabidopsis*, where methylation is directed to specific segments, for example, the tandem repeats in the promoter of *FWA* (Kinoshita et al. 2004, 2007) and a 200-bp upstream segment in *FIS2*, respectively (Jullien and Katz et al. 2006; Jullien and Kinoshita et al. 2006). There are no direct or inverted repeats, which could act as *cis*-acting methylation elements in the *Fie1* gene. Recently a DMR (Differentially Methylated Region) was identified in *Fie1* at the 5' promoter region and in exon 1. However, in the central cell and the 6 DAP endosperm, DNA methylation was detected only in the promoter segment of the DMR but not in exon 1 and other downstream sequences (Gutierrez-Marcos et al. 2006). In contrast, we have detected that the paternal *Fie1* allele is methylated over the same extended region including the promoter, exon 1 and exons 6–8 in the 14 DAP endosperm as it was in the pollen. The different methylation pattern of *Fie1* at 6DAP (Gutierrez-Marcos et al. 2006) and 14 DAP endosperm (this report) may reflect the complex dynamics of demethylation and de novo methylation of the paternal *Fie1* allele that occurs during endosperm development in maize. Our study of the imprinted *Fie1* gene in maize provides new evidence for diversity and adds further complexity of imprinting mechanisms in plants.

Acknowledgements We thank Evgueni Ananiev and Mike Muszynski for numerous critical comments, Dan Spielbauer and Laura Gottschalk for help with a real-time PCR experiment as well as Kejian Li and Anna Lyznik for technical assistance. This work was supported by a post-graduate scholarship in accordance with Hamburg's Young Academics Funding Law to K.S.

References

Alleman M, Doctor J (2000) Genomic imprinting in plants: observations and evolutionary implications. Plant Mol Biol 43(2–3):147–161

Baroux C, Gagliardini V et al (2006) Dynamic regulatory interactions of Polycomb group genes: MEDEA autoregulation is required for imprinted gene expression in Arabidopsis. Genes Dev 20(9):1081–1086

Baroux C, Spillane C et al (2002) Genomic imprinting during seed development. Adv Genet 46:165–214

Choi Y, Gehring M et al (2002) DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in arabidopsis. Cell 110(1):33–42

Cordts S, Bantin J et al (2001) ZmES genes encode peptides with structural homology to defensins and are specifically expressed in the female gametophyte of maize. Plant J 25(1):103–114

Danilevskaya ON, Hermon P et al (2003) Duplicated fie genes in maize: expression pattern and imprinting suggest distinct functions. Plant Cell 15(2):425–438

Delaval K, Feil R (2004) Epigenetic regulation of mammalian genomic imprinting. Curr Opin Genet Dev 14(2):188–195

Dilkes BP, Dante RA et al (2002) Genetic analyses of endoreduplication in Zea mays endosperm: evidence of sporophytic and zygotic maternal control. Genetics 160(3):1163–1177

Dresselhaus T, Cordts S et al (1999) Novel ribosomal genes from maize are differentially expressed in the zygotic and somatic cell cycles. Mol Gen Genet 261(2):416–427

Engemann S, El-Maarri O et al (2001) Bisulfite-based methylation analysis of imprinted genes. Methods Mol Biol 181:217–228

Gehring M, Choi Y et al (2004a) Imprinting and seed development. Plant Cell 16:S203–S213

Gehring M, Choi Y et al (2004b) Imprinting and seed development. Plant Cell 16(Suppl):S203–S213

Gehring M, Huh JH et al (2006) DEMETER DNA glycosylase establishes MEDEA polycomb gene self-imprinting by allele-specific demethylation. Cell 124(3):495–506

Grossniklaus U, Schneitz K (1998) The molecular and genetic basis of ovule and megagametophyte development. Semin Cell Dev Biol 9(2):227–238

Grossniklaus U, Vielle-Calzada JP et al (1998) Maternal control of embryogenesis by MEDEA, a polycomb group gene in Arabidopsis. Science 280(5362):446–450

Gutierrez-Marcos JF, Costa LM et al (2006) Epigenetic asymmetry of imprinted genes in plant gametes. Nat Genet 38(8):876–878

Gutierrez-Marcos JF, Pennington PD et al (2003) Imprinting in the endosperm: a possible role in preventing wide hybridization. Philos Trans R Soc Lond B Biol Sci 358(1434):1105–1111

Ingouff M, Haseloff J et al (2005) Polycomb group genes control developmental timing of endosperm. Plant J 42(5):663–674

Jullien PE, Katz A et al (2006) Polycomb group complexes self-regulate imprinting of the Polycomb group gene MEDEA in Arabidopsis. Curr Biol 16(5):486–492

Jullien PE, Kinoshita T et al (2006) Maintenance of DNA Methylation during the Arabidopsis life cycle is essential for parental imprinting. Plant Cell 18(6):1360–1372

Kermicle JL (1970) Dependence of the R Mottled Aleurone phenotype in maize-M on mode of sexual transmission. Genetics 66(1):69–85

Kiesselbach TA (1999) The structure and reproduction of corn. 50th aniversary edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Kinoshita T, Miura A et al (2004) One-way control of FWA imprinting in Arabidopsis endosperm by DNA methylation. Science 303(5657):521–523

Kinoshita T, Yadegari R et al (1999) Imprinting of the MEDEA polycomb gene in the Arabidopsis endosperm. Plant Cell 11(10):1945–1952

Kinoshita Y, Saze H et al (2007) Control of FWA gene silencing in *Arabidopsis thaliana* by SINE-related direct repeats. Plant J 49(1):38–45

Köhler C, Hennig L et al (2003a) Arabidopsis MSI1 is a component of the MEA/FIE Polycomb group complex and required for seed development. Embo J 22(18):4804–4814

Köhler C, Hennig L et al (2003b) The Polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene PHERES1. Genes Dev 17(12):1540–1553

Köhler C, Page DR et al (2005) The *Arabidopsis thaliana* MEDEA Polycomb group protein controls expression of PHERES1 by parental imprinting. Nat Genet 37(1):28–30

Kowles RV, Phillips RL (1988) Endosperm development in maize. Int Rev Cytol 112:97–136

Kranz E, von Wiegen P et al (1998) Endosperm development after fusion of isolated, single maize sperm and central cells in vitro. Plant Cell 10(4):511–524

Lai J, Ma J et al (2004) Gene loss and movement in the maize genome. Genome Res 14(10A):1924–1931

Lauria M, Rupe M et al (2004) Extensive maternal DNA hypomethylation in the endosperm of Zea mays. Plant Cell 16(2):510–522

Leblanc O, Pointe C et al (2002) Cell cycle progression during endosperm development in Zea mays depends on parental dosage effects. Plant J 32(6):1057–1066

Liang HE, Hsu LY et al (2004) Variegated transcriptional activation of the immunoglobulin kappa locus in pre-b cells contributes to the allelic exclusion of light-chain expression. Cell 118(1):19–29

Luo M, Bilodeau P et al (1999) Genes controlling fertilization-independent seed development in Arabidopsis thaliana. Proc Natl Acad Sci USA 96(1):296–301

Morison IM, Ramsay JP et al (2005) A census of mammalian imprinting. Trends Genet 21(8):457–465

Ohad N, Yadegari R et al (1999) Mutations in FIE, a WD polycomb group gene, allow endosperm development without fertilization. Plant Cell 11(3):407–416

Reik W, Walter J (2001) Genomic imprinting: parental influence on the genome. Nat Rev Genet 2(1):21–32

Richert J, Kranz E, Lörz H, Dresselhaus T (1996) Plant Sci 114:93–99

Sathe SS, Harte PJ (1995) The Drosophila extra sex combs protein contains WD motifs essential for its function as a repressor of homeotic genes. Mech Dev 52(1):77–87

Springer NM, Danilevskaya ON et al (2002) Sequence relationships, conserved domains, and expression patterns for maize homologs of the polycomb group genes E(z), esc, and E(Pc). Plant Physiol 128(4):1332–1345

Surani MA, Barton SC (1983) Development of gynogenetic eggs in the mouse: implications for parthenogenetic embryos. Science 222(4627):1034–1036

Walbot V, Evans MM (2003) Unique features of the plant life cycle and their consequences. Nat Rev Genet 4(5):369–379

Xiao W, Gehring M et al (2003) Imprinting of the MEA Polycomb gene is controlled by antagonism between MET1 methyltransferase and DME glycosylase. Dev Cell 5(6):891–901